Sodium Channels Aggregate at Former Synaptic Sites in Innervated and Denervated Regenerating Muscles

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Abstract. The role of innervation in the establishment and regulation of the synaptic density of voltage-activated Na channels (NaChs) was investigated at regenerating neuromuscular junctions. Rat muscles were induced to degenerate after injection of the Australian tiger snake toxin, notexin. The loose-patch voltage clamp technique was used to measure the density and distribution of NaChs on muscle fibers regenerating with or without innervation. In either case, new myofibers formed within the original basal lamina sheaths, and, NaChs became concentrated at regenerating endplates nearly as soon as they formed. The subsequent increase in synaptic NaCh density followed a time course similar to postnatal muscles. Neuromuscular endplates regenerating after denervation, with no nerve terminals present, had NaCh densities not significantly different from endplates regenerating in the presence of nerve terminals. The results show that the nerve terminal is not required for the development of an enriched NaCh density at regenerating neuromuscular synapses and implicate Schwann cells or basal lamina as the origin of the signal for NaCh aggregation. In contrast, the change in expression from the immature to the mature form of the NaCh isoform that normally accompanies development occurred only partially on muscles regenerating in the absence of innervation. This aspect of NaCh regulation is thus dependent upon innervation.

VOLTAGE-activated sodium channels (NaChs)[1] are important for propagation of the action potential in excitable membrane of nerve and muscle (for review see Catterall, 1992). NaChs are distributed uniformly over most of the surface membrane of adult skeletal muscle fibers, but are concentrated ~10-fold in the postsynaptic membrane (Betz et al., 1984; Beam et al., 1985; Angelides, 1986; Caldwell et al., 1986; Roberts, 1987). Although the synaptic distribution of NaChs appears similar to that of acetylcholine receptors (AChRs) and several other synaptic molecules (for review see Froehner, 1993), previous results indicate that the processes regulating the distributions of NaChs and AChRs are different. For example, the synaptic cluster of AChRs forms about one week before birth in rodents, while the concentration of NaChs begins to appear at endplates around one week after birth (Lupa et al., 1993). The endplate density of NaChs increases gradually over the first 5–6 wk after birth. Also, the basal lamina protein agrin, which is believed to play a primary role in AChR clustering, had little effect on the distribution of NaChs on muscle cells in culture (Lupa and Caldwell, 1991). Finally, ultrastructural studies have revealed that NaChs and AChRs occupy distinct domains in the postsynaptic membrane, with AChRs at the top of the junctional folds and NaChs in the lower half of the synaptic troughs (Flucher and Daniels, 1989; Boudier et al., 1992).

Our goal is to understand the mechanism by which NaChs become stably aggregated at neuromuscular endplates. In the present study we have conducted experiments to test whether the nerve terminal is a necessary component for NaCh aggregation to occur at regenerating neuromuscular junctions. Muscle degeneration and regeneration was induced with the snake toxin notexin (Harris et al., 1975; Harris and Cullen, 1990), and the distribution of NaChs on regenerating fibers was monitored with the loose-patch voltage clamp technique (Strickholm, 1961; Almers et al., 1983). Specifically, we have asked two questions: (1) will NaChs aggregate at endplates of muscle fibers regenerating after injury, and (2) will NaChs aggregate even in the absence of innervation? The results showed that NaChs aggregate on regenerating muscle fibers, either at the same time or soon after formation of the AChR patch at the new endplate. This aggregation of NaChs could occur on regenerating muscle fibers even in the absence of innervation. We conclude that a signal that can direct NaCh aggregation must reside in the basal lamina or originate from the Schwann cells that remain after muscle degeneration.

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; CGRP, calcitonin gene-related peptide; FDB, flexor digitorum brevis; NaCh, voltage-activated sodium channel; TTX, tetrodotoxin.

Two types of NaChs are expressed in muscle cells, and they are the products of different genes (Redfern and Thesleff, 1971; Trimmer et al., 1989; Kallen et al., 1990). One type is expressed primarily in adult innervated muscle and is effectively blocked by low concentrations of tetrodotoxin (TTX), with a K_D of ~10 nM (TTX-sensitive or TTX-S NaCh). A second NaCh subtype (TTX-resistant or TTX-R NaCh) is expressed in developing and denervated muscle and is relatively resistant to the blocking effects of TTX, with a K_D of ~1 μM. It has recently been shown that synaptic basal lamina could direct the switch in expression from embryonic to adult AChR subunits in regenerating muscles, even when the nerve was absent during regeneration (Goldman et al., 1991; Brenner et al., 1992). We used TTX to estimate the populations of TTX-R and TTX-S NaChs present at regenerating endplates in the absence or presence of nerve terminals. The switch in expression from TTX-R to TTX-S NaChs only partially occurred in denervated muscles, suggesting that some aspect of innervation was required for the developmental switch in NaCh expression to occur.

Materials and Methods

Regenerated Muscle Preparation

The muscle preparation used was the rat flexor digitorum brevis (FDB) muscle. FDB muscles were removed from rats after an overdose of ether and dissociated into single fibers after incubation in 0.25% collagenase B (Boehringer-Mannheim, Indianapolis, IN) and 0.1% BSA (Sigma Chem. Co., St. Louis, MO) for 2 h (Bekoff and Betz, 1977).

Degeneration and subsequent regeneration was induced by a single injection into the footpad of 2.6 μg (in 0.16 ml saline) of the notexin fraction (Latoxan, France) of venom from the Australian tiger snake *Notechis scutatus scutatus* (Harris and Cullen, 1990). This amount of toxin causes total degeneration of the muscle within 48 h, leaving behind only the basal lamina and associated fibroblasts, myoblasts, Schwann cells, and satellite cells (Harris et al., 1975; Grubb et al., 1991). Complete degeneration of FDB muscles was confirmed in several experiments by dissociation of muscles 2–4 d after toxin injection. At this early time only mononucleated cells could be extracted from the mass of connective tissue that made up the muscle.

For regeneration in the absence of innervation, rats were denervated unilaterally under ether anesthesia by cutting the nerve in mid-thigh, followed 1–2 d later by toxin injection into both footpads. Time of reinnervation was monitored by appearance of the toe-spreading reflex (absent in denervated muscles) as well as elicitation of a twitch when cutting the nerve during dissection. By these criteria, muscles remained denervated for ~4 wk after this procedure.

Notexin has been shown to damage nerve terminals in addition to its myotoxic effects. Muscles injected with notexin, but not denervated by nerve section, showed a muscle twitch at the earliest times the muscle could be removed (5–6 d after injection). Moreover, Grubb et al. (1991) were able to record miniature end plate potentials from all synapses by 5 d after injection of notexin. These results show that any nerve terminal damage induced by notexin did not last more than a few days.

Neuromuscular endplates were visualized after labeling AChRs with rhodamine-conjugated α-bungarotoxin (rho-Butx), prepared according to Ravdin and Axelrod (1977). Preparations were viewed on a Nikon Diaphot inverted microscope and images were captured through a Dage SIT camera interfaced with a Silicon Graphics (Mountain View, CA) Iris computer, as described previously (Lupa and Caldwell, 1991).

Loose-Patch Voltage Clamp Recording

Na current measurements were made using the loose-patch voltage clamp technique (Strickholm, 1961; Almers et al., 1983), as previously described (Lupa and Caldwell, 1991). Briefly, lightly fire-polished micropipettes of 6–12 μm tip diameter were filled with Ringer solution and pushed against the surface of muscle fibers using an Inchworm piezoelectric controller (Burleigh Instruments, Fishers, NY) set at a 2-μm step. Slight suction (10–15 mm Hg) was applied to improve the electrical seal. Current measurements were used if the seal resistance was at least twice the electrode resistance (150–400 Kohm). Recordings were made with a constant potential (70 mV) applied through the electrode. This was applied for 1–2 min before recording the maximum inward current and made the membrane potential of the patch 70 mV more negative than the resting potential. This was done in order to reduce slow-inactivation of NaChs (Almers et al., 1983). Na currents were photographed directly from the oscilloscope with a Polaroid C-5C camera, and normalized by the area under the pipette tip to calculate Na current density (see Lupa et al., 1993).

Pharmacological blockade by TTX was used to estimate the amount of Na current produced by the mature (TTX-S) and immature (TTX-R) forms of the NaCh. Identified single fibers were recorded in normal Ringer solution, then relocated by stage micrometer settings after addition of 300 nM TTX. The solution in the recording pipette was also changed to prevent dilution of the toxin at the membrane patch. Assuming K_Ds of 1 μM and 10 nM for TTX-R and TTX-S NaChs, respectively, we calculate that this concentration of TTX will block ~23% of the TTX-R NaChs along with 97% of the TTX-S NaChs. Measurements of Na current obtained in normal Ringer and in 300 nM TTX were used to estimate the amount of current attributable to the TTX-R and TTX-S NaChs by the equation $NaI_{TTX} = (NaI_{TOX} - 0.03(NaI_{TOT}))/0.74$, where NaI_{TOT} is the current recorded in normal Ringer solution, NaI_{TOX} is the uncorrected current measured in the presence of 300 nM TTX, and NaI_{TTX} is the Na current through TTX-R NaChs.

Statistics

All current density measurements are given as the mean ± SEM; statistical significance was tested with students' *t* test for unpaired parameters.

Results

NaCh Distribution on Regenerating Muscle Fibers in Innervated Muscles

Local injection of notexin into mammalian muscles induces necrosis and complete degeneration of muscle fibers within 24–48 h (Harris et al., 1975; Brenner et al., 1992). Axons and muscle fibers are damaged and phagocytized by macrophages, while the Schwann cells, basal lamina sheaths, and microvasculature are left intact. Satellite cells, which are evidently not affected by the toxin, begin to proliferate after 2–3 d and start to form myotubes on the fourth day after toxin injection (Harris et al., 1975; Grubb et al., 1991).

The earliest time that FDB muscles could be successfully removed and dissociated into single fibers was 5 d after injection (Fig. 1). At this time myofibers consisted of thin myotubes (<10 μm diam) with numerous myoblasts attached in various stages of fusion along the length of the fiber, resembling pearls on a string. When labeled with rho-Butx to visualize AChRs, no endplate region could be discerned. Within 36 h, however (6–7 d after notexin), a distinct oval area of high AChR density could be identified, signaling the differentiation of the neuromuscular endplate (see also Grubb and Harris, 1989; Whalen et al., 1990). Often this rho-Butx labeling appeared to be restricted to the domains of one or two nuclei that bulged out from the muscle cell as if they had recently fused into the myotube. Ten days after notexin, the rho-Butx labeling at the endplate appeared essentially normal, and by 15 d the muscle cells had developed prominent cross-striations (Fig. 1).

The distribution of NaChs on regenerating muscle fibers was assayed with the loose-patch clamp technique. Recordings were made with the pipette directly over the endplate and on extrajunctional membrane (see Figs. 2 and 3). Na current densities recorded from regenerated muscles 6–7 d

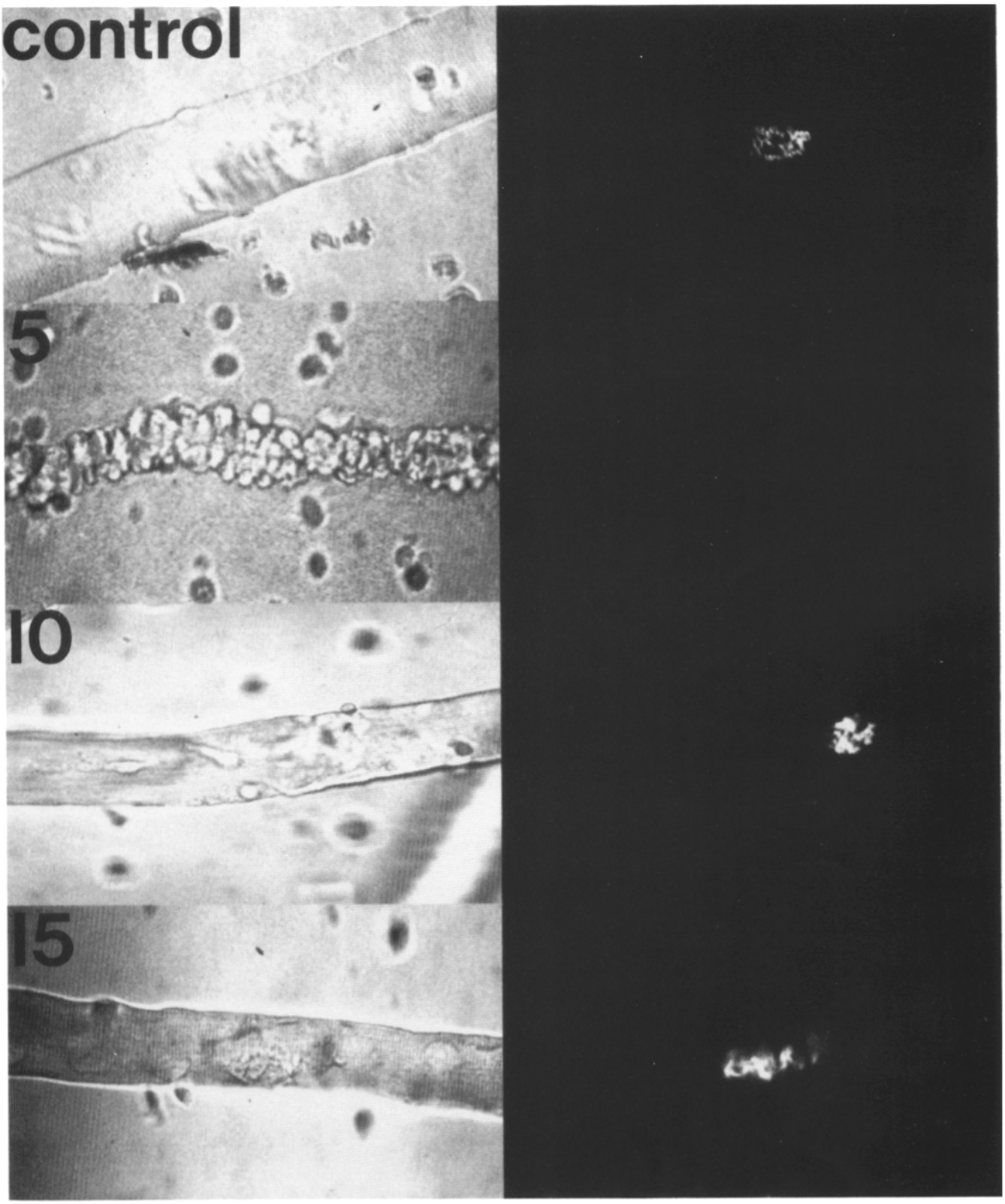

Figure 1. Images of a control innervated FDB muscle fiber and regenerating muscle fibers 5, 10, and 15 d after injection of notexin. On the left are Hoffman phase contrast images and on the right are rhodamine fluorescence images showing the endplates labeled with rho-Butx. On muscles taken 5 d after toxin injection no distinct area of rho-Butx fluorescence resembling a neuromuscular endplate could be detected. By 10–15 d after notexin the muscle fibers appeared essentially normal, with a prominent neuromuscular endplate region.

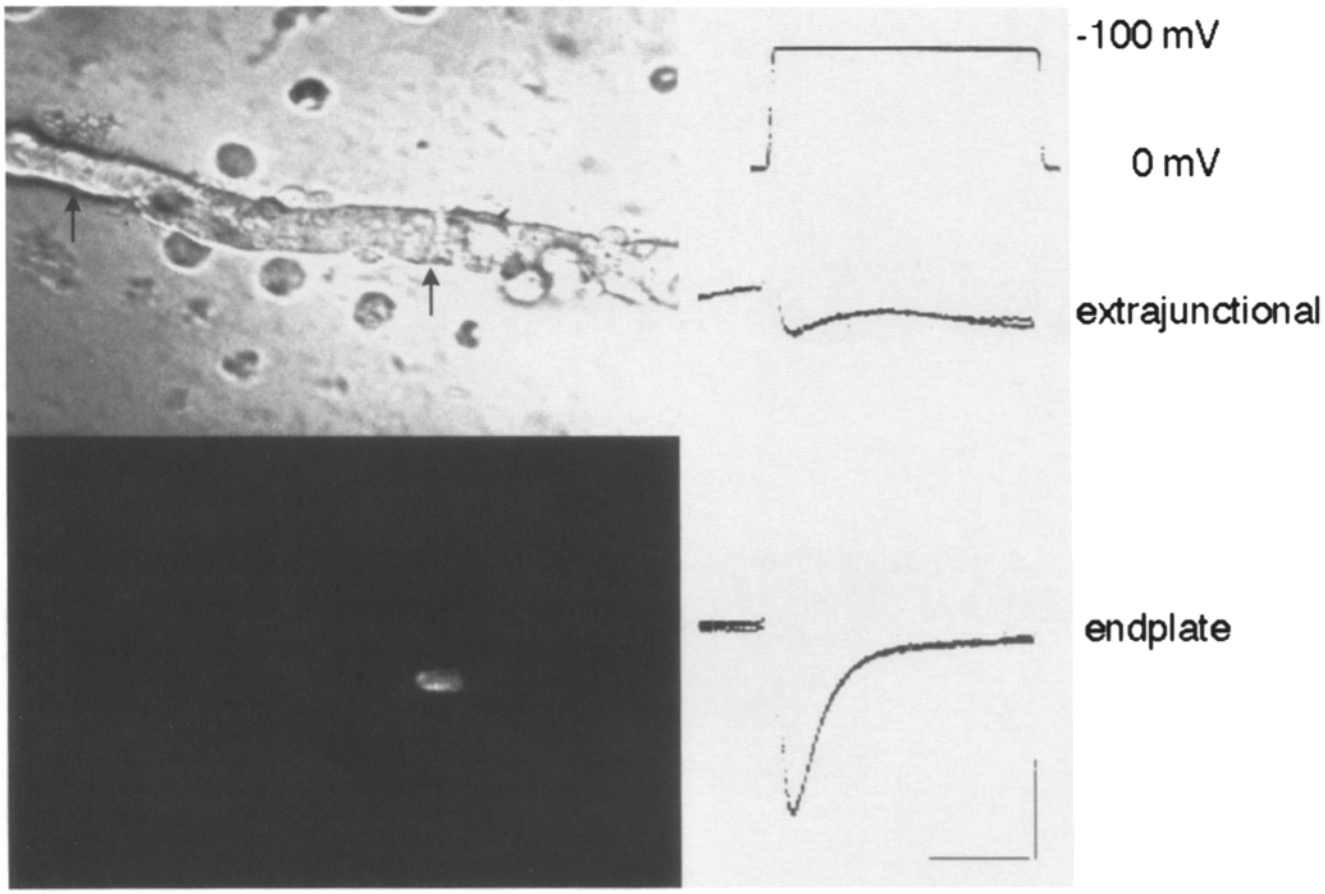

Figure 2. The left panels are a Hoffman phase contrast (*top*) and a rho-Butx fluorescence (*bottom*) image of a muscle fiber dissociated from an FDB muscle 7 d after injection of notexin. Arrows show positions of patch electrode during recording. The right panels are recordings of Na current made with the loose-patch electrode directly over the endplate (*bottom*) or on extrajunctional membrane (*middle*) 150 μm away from the endplate. Na current density was already enriched 3.5-fold at the endplate by this time. Calibration bars are 15 mA/cm^2 and 1 ms.

after injection of notexin, when the endplate patch of AChRs first appeared, revealed that NaChs became concentrated at regenerated endplates nearly as soon as they formed. The NaCh distribution at this time resembled the distribution on muscles taken from newborn rats during the second week after birth (Lupa et al., 1993). Na current density at endplates of 6 d regenerated muscle fibers was 18.2 $\pm$ 2.0 mA/cm^2, and extrajunctional current density was 8.7 $\pm$ 1.6 mA/cm^2 ($p < 0.01$; Fig. 3). It is possible that at this early time the measurement of endplate Na current was higher than extrajunctional current because of the reappearance of post junctional folds (Brenner et al., 1992). The further increase, however, could not be explained by increased membrane area (see Lupa et al., 1993). The NaCh concentration increased gradually in endplate membrane and decreased in extrajunctional membrane after longer periods of regeneration, again as in postnatal muscles (Lupa et al., 1993). By 13 d after toxin injection, NaChs were concentrated ~4.5-fold at the endplate, with the current density in endplate and extrajunctional membrane being 30.0 $\pm$ 3.2 mA/cm^2 and 7.4 $\pm$ 0.8 mA/cm^2, respectively. The NaCh density at the endplate continued to increase until 23 d after notexin, when it was 65.0 $\pm$ 9.0 mA/cm^2.

These experiments showed that NaChs become concentrated at endplates of new muscle fibers regenerating after an injection of notexin. This process lasted several weeks, with a time course similar to postnatal development. The next question was whether this process could occur in the absence of the nerve terminal.

NaCh Distribution on Regenerating Muscle Fibers in Denervated Muscles

Muscle regeneration after the necrosis caused by notexin occurred similarly whether the innervation was cut or left intact. The only difference evident was the atrophy of fibers regenerating in the absence of innervation (see also Whalen et al., 1990; Brenner et al., 1992), reminiscent of atrophy seen in chronically denervated muscle fibers (Jirmanova et al., 1964; Albuquerque and Thesleff, 1968). Neuromuscular endplates, as judged by rho-Butx fluorescence, appeared in denervated muscles with the same time course as in innervated muscles (Burden et al., 1979; Brenner et al., 1992).

Na currents recorded from endplates of regenerating muscles also appeared with the same time course regardless of the state of innervation (Fig. 4). During the early stages of regeneration there was no significant difference in the NaCh distribution of muscles regenerating in the presence or ab-

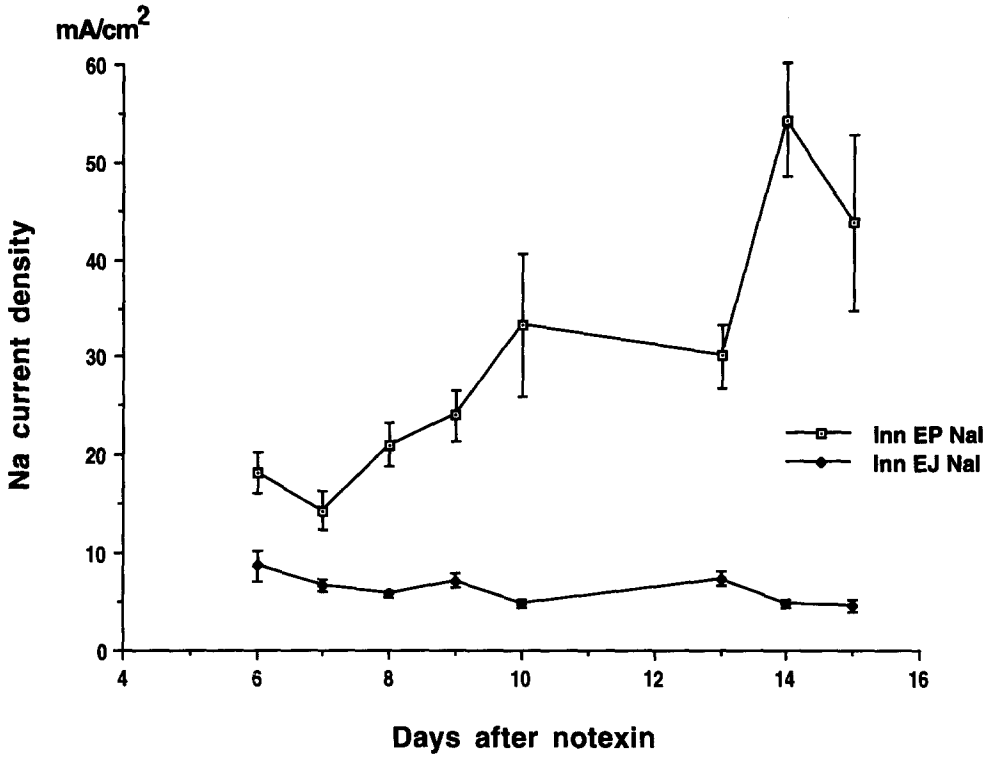

Figure 3. NaChs aggregate at endplates of muscles regenerating after notexin poisoning. Na current density measured from endplate (*open squares*) and extrajunctional (*closed diamonds*; 100–200 μm from the endplate) membrane of FDB muscles regenerating in the presence of innervation. Na current density was significantly higher in endplate compared to extrajunctional membrane at the earliest time that an endplate could be discerned (5–6 d after notexin injection). Over the next 10 d the Na current density in extrajunctional membrane became gradually reduced, while Na current density at the endplate increased 3–4-fold.

sence of innervation. At the earliest time that an endplate could be visualized with rho-Butx, Na currents were higher at the endplate than in extrajunctional membrane. In denervated muscles studied 6 d after notexin poisoning the Na current density at endplates was 16.2 ± 3.0 mA/cm², while extrajunctional current density in these muscle fibers was 10.0 ± 0.7 mA/cm² ($p < 0.05$). This non-uniform distribution of NaChs developed with a time course similar to muscles regenerating in the presence of innervation, and after 13 d of regeneration, the Na current density in endplate and extrajunctional membrane was 32.3 ± 3.2 mA/cm² and 7.0 ± 0.7 mA/cm², respectively. These results show that develop-

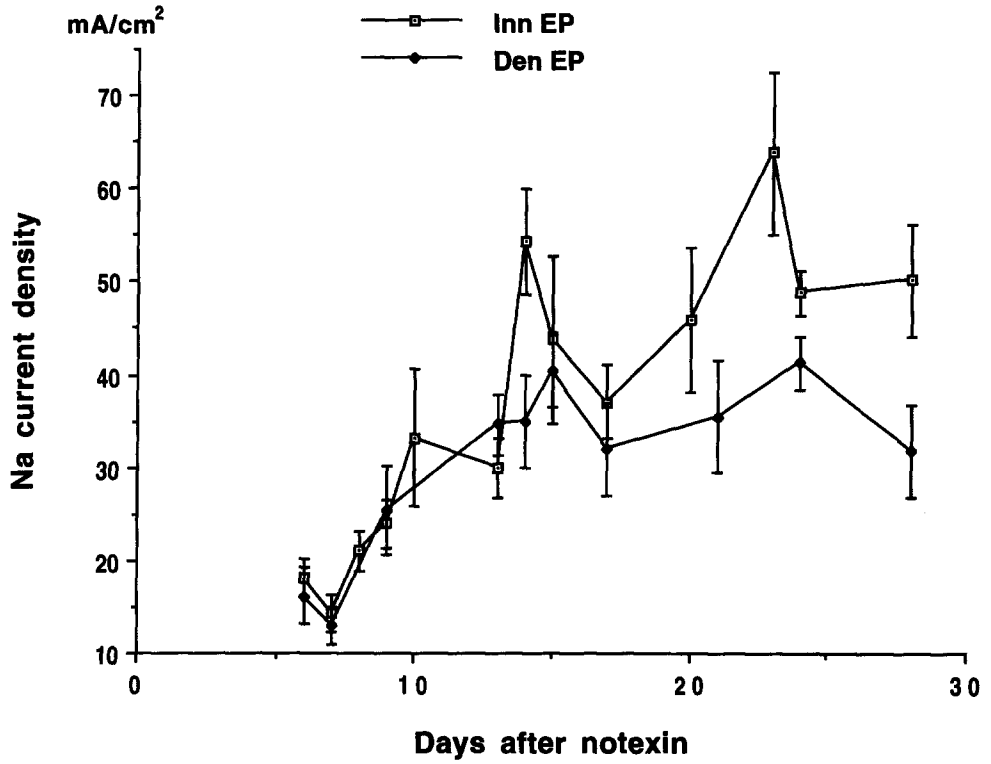

Figure 4. NaChs aggregate at endplates of regenerating muscle fibers in the absence of the nerve terminal. No significant difference was noted between Na current densities at endplates in denervated (*closed diamonds*) or innervated (*open squares*) regenerating muscles until after day 13. From this time on, the endplate current density continued to increase in innervated muscles, but remained relatively constant in denervated muscles.

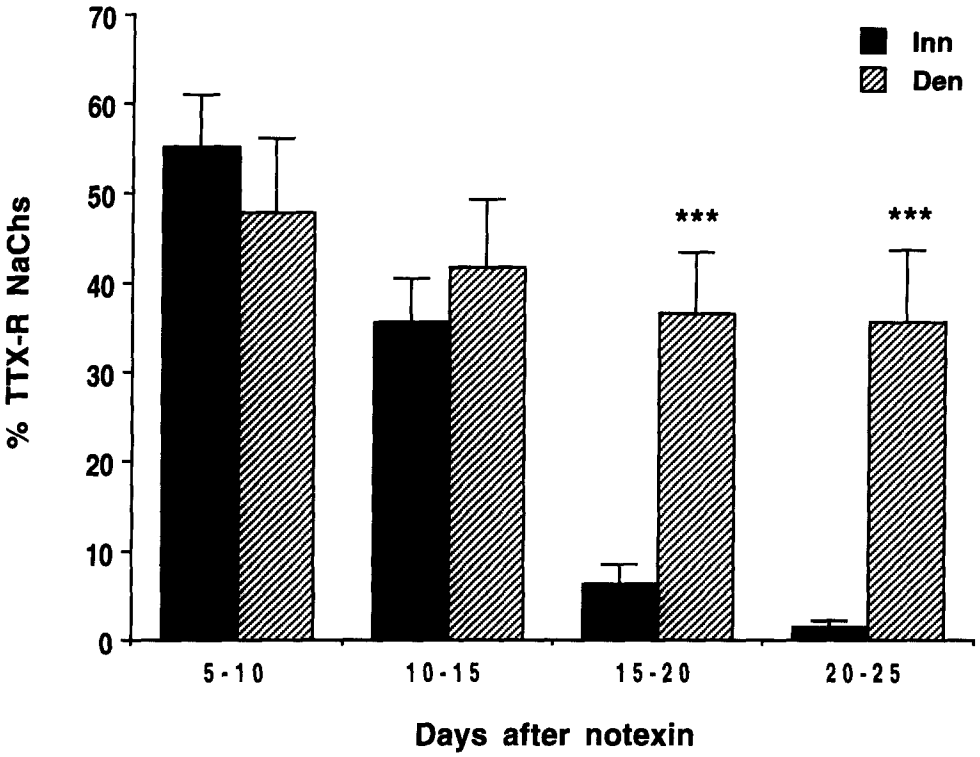

Figure 5. Change in the percentage of TTX-R NaChs at endplates of regenerating muscle fibers. Bars show the percentage of Na current density attributable to the TTX-R NaChs, calculated as described in the Methods, at various times of regeneration. Solid bars are from innervated muscles, hatched bars are from denervated muscles. ***, $p < 0.001$.

ment of a high NaCh concentration at endplates of regenerating muscle fibers occurs even in the absence of innervation and therefore must be initiated by the Schwann cells or basal lamina that remain after muscle and nerve degeneration.

At later times of regeneration (>15 d), Na current density at innervated endplates appeared to be slightly, but consistently, higher than at endplates regenerating in the absence of innervation. This difference was small (~30%) and marginally significant at any time point. Pooled data from muscles regenerating for 20–28 d in the presence of innervation revealed an average endplate current density of 49.7 ± 3.5 mA/cm² (n = 24), while the contralateral muscles regenerating in the absence of innervation had a mean Na current density of 33.8 ± 2.9 mA/cm² (n = 28; $p < 0.01$). These results may reflect an influence of innervation on the later stages of NaCh aggregation.

Changes in NaCh Isoform during Muscle Regeneration

A change in the NaCh isoform expressed by rodent muscle cells occurs during postnatal development (Trimmer et al., 1990; Lupa et al., 1993). Just after birth the TTX-R, immature form of the NaCh predominates, accounting for ~90% of the Na current density (Gonoi et al., 1989; Lupa et al., 1993). Gradually, expression of the immature NaCh subtype is reduced while expression of the mature NaCh increases. Since the immature and mature forms of the NaCh can be distinguished by their affinity for TTX, this toxin was used as a tool to separate electrophysiologically the two populations of NaChs. It has been shown that factors in the basal lamina can induce synapse-specific expression of adult AChR subunit genes (Goldman et al., 1991; Brenner et al., 1992), and it seemed possible that a similar mechanism could direct NaCh isoform expression at regenerating neuromuscular endplates.

FDB muscles were denervated unilaterally one or two days before injecting notexin into both footpads. Na currents were recorded at endplates in normal Ringer and in Ringer with 300 nM TTX. Fig. 5 shows the percentage of endplate current through TTX-R NaChs calculated at different times of regeneration (see Methods). Over the first two weeks of regeneration the percentage of the endplate NaCh population made up by TTX-R NaChs gradually decreased to near 40%, and there was no significant difference between innervated and previously denervated muscle. During the third week of regeneration, however, the percentage of TTX-R NaChs decreased to 6.3 ± 2.3% at innervated endplates while remaining significantly higher (36.7 ± 6.7%) at denervated endplates ($p < 0.001$). The shift in expression from the immature, TTX-R to the mature, TTX-S form of the NaCh thus appeared to occur in two distinct phases. During the first phase, which was independent of innervation, the population of TTX-R NaChs decreased to ~40% of the total population. The second phase, during which endplates acquired their adult densities of TTX-R and TTX-S NaChs, occurred only in the presence of the nerve.

Discussion

The results demonstrate that aggregation of NaChs at synapses of regenerating muscle cells occurs even in the absence of nerve terminals and must be directed by factors that remain after denervation of the muscle. In contrast, the majority of the change in NaCh isoform that normally occurs during maturation of the neuromuscular endplate requires the presence of nerve terminals.

The present results also reinforce the evidence that NaCh aggregation is a relatively slow process that is a part of synaptic maturation at the neuromuscular junction. The density

of NaChs at regenerating endplates increased gradually over several weeks, as observed during postnatal development (Lupa et al., 1993). The onset of NaCh aggregation corresponds with the appearance of postjunctional folding in both postnatal and regenerating muscles. Elaborate infolding of the muscle fiber membrane occurs in the region of the synaptic sites within 1–2 d of new muscle fiber formation after notexin treatment, even in the absence of innervation (Brenner et al., 1992). The results suggest that the molecular signals left after removal of muscle fibers and nerve terminals can direct several aspects of synaptic maturation.

Mechanism of NaCh Aggregation

The destruction of muscle fibers is extensive after notexin treatment and involves the loss of all the original muscle fiber membrane and myonuclei (see Harris and Cullen, 1990; Brenner et al., 1992). The only elements remaining after denervation and notexin poisoning are myoblasts, Schwann cells, the basal lamina sheath, and remnants of the degenerating nerve terminal (Burden et al., 1979; Harris and Cullen, 1990; Slater, 1990). Any one or all of these elements could be involved in the mechanism inducing the early aggregation of NaChs at developing neuromuscular endplates. It is possible that Schwann cells, which normally overlie the nerve terminal and move into close apposition with the neuromuscular endplate after denervation, might provide the signal for NaCh aggregation, either through a contact-mediated or secreted molecular signal. Schwann cells have been shown to induce clustering of NaChs on cultured neurons (Joe and Angelides, 1992), and to facilitate synaptic maturation at neuromuscular contacts in culture (Chapron and Koenig, 1989). The potential role of Schwann cells can be tested in two ways. Schwann cells could be eliminated by freezing the muscle at the same time as it is denervated (McMahan and Slater, 1984; Brenner et al., 1992). Subsequent muscle regeneration would occur in the absence of both nerve terminals and Schwann cells. Aggregation of NaChs in this situation would effectively rule out Schwann cells as a source of NaCh aggregating activity. Alternatively, Schwann cells and muscle fibers could be cocultured and the ability of Schwann cells to induce NaCh clusters at sites of contact with muscle fibers could be measured immunocytochemically (see Lupa et al., 1993) and with loose-patch voltage clamp.

The most likely source for the molecular signals directing regeneration of the neuromuscular endplate is the basal lamina. This molecular sheath, which normally tightly surrounds the muscle fiber and Schwann cell, remains intact and retains a high concentration of acetylcholinesterase, agrin, and other molecules even after degeneration of the nerve terminal and muscle fiber (McMahan et al., 1978; Mège et al., 1992; Brenner et al., 1992). McMahan and colleagues have shown that the agrin protein, which was originally purified from an extract of *Torpedo* electric organ, is the molecule primarily responsible for aggregation of AChRs at regenerating, and most likely, developing neuromuscular synapses (McMahan et al., 1992). Previous results have shown that *Torpedo* agrin does not induce clustering of NaChs after up to 60 h of exposure (Lupa and Caldwell, 1991). These experiments, at the time, seemed to rule out a direct role for agrin in NaCh clustering. However, since the NaCh aggregation process appears to be a gradual one that becomes apparent only after several days to weeks of development (Fig. 3; Lupa et al., 1993), a slow effect of agrin on NaChs might have been missed in this previous study. Furthermore, since several forms of agrin are expressed in rats (Ferns et al., 1992; Ruegg et al., 1992), it may be that some forms of agrin are active in AChR clustering, with other forms active in NaCh clustering or other aspects of synaptic development (Ferns and Hall, 1992). This scenario is bolstered by the recent report that the alternative splicing isoforms of agrin are developmentally regulated (Hoch et al., 1993). In the spinal cord, where motor neurons synthesize agrin, one agrin isoform increases after birth, around the time that NaChs begin to aggregate. In preliminary experiments we have tested two of these agrin isoforms, $agrin_0$ and $agrin_8$ (Ferns et al., 1992), without detecting evidence of NaCh aggregation after up to 6 d of culture (Lupa, M. T., J. T. Campanelli, R. H. Scheller, and J. H. Caldwell, unpublished data). It is possible that 6 d in culture may not be long enough to observe the effect of agrin on NaCh distribution. Although all the tests of agrin's direct effect on NaCh clustering have been negative, none of them rules out a role for agrin. It is also possible that agrin plays an indirect role, triggering a series of events, some of which are rapid and others more prolonged. Since the spatial and temporal clustering of AChRs are so different from NaChs, it is likely that undiscovered molecules, in addition to agrin, act to mediate events in synaptic maturation, including aggregation of NaChs at the neuromuscular junction.

After ~15 d of regeneration a difference appeared in NaCh density at endplates of denervated and innervated muscles. While Na current density at innervated endplates continued to increase, the current density on denervated muscle fibers changed comparatively little. After 28 d of regeneration in the absence of innervation, for instance, the Na current density was 31.8 $\pm$ 5.0 mA/cm^2; this was 36% less than the corresponding Na current density (50.0 $\pm$ 6.0) in contralateral muscles regenerating in the presence of nerve terminals. This difference was only marginally significant, but it suggests that a later phase of NaCh aggregation might be dependent upon the presence of the nerve terminal.

Control of NaCh Isoform at Regenerating Endplates

It has recently been reported that the basal lamina contains all the components necessary to direct local expression of the mature form of the acetylcholine receptor at the neuromuscular endplate (Goldman et al., 1991; Brenner et al., 1992). It seemed possible, therefore, that the switch in expression from the TTX-R to the TTX-S isoform of the NaCh could also be directed by the basal lamina at regenerating endplates. The results cited above for AChR aggregation are qualitatively different from those reported here for the NaCh. A small component of the NaCh population did change from TTX-R to TTX-S even in the absence of the nerve. However, the present results demonstrate that the presence of the nerve terminal is necessary for the majority of this switch to occur. This may be taken as further evidence that different regulatory signals are controlling the densities of acetylcholine receptors and NaChs at the neuromuscular junction.

The disappearance of the TTX-R isoform and appearance of the TTX-S NaCh subtype occurred in two phases. During the first two weeks of regeneration there was no significant

difference in the density of TTX-R NaChs at innervated or denervated regenerating endplates. At the earliest times studied (5–10 d after notexin) ~50% of the population of endplate NaChs was made up of the TTX-R isoform, regardless of innervation. This percentage decreased to ~40% during the second week of regeneration. During the third week of regeneration the population of TTX-R NaChs decreased dramatically to ~6% of the total at innervated endplates while remaining at ~37% of the NaCh population at denervated endplates (Fig. 5). These results resemble those reported for the appearance of saxitoxin-binding sites on postnatal muscles (Sherman and Catterall, 1982). In those experiments the regulation of TTX-S NaCh density was shown to be biphasic. The early phase, from birth to postnatal day 11, was shown to occur independently of innervation and increased TTX-S NaCh density to ~55% of the normal adult level. This phase would correspond to the first two weeks of regeneration described in the present results. The second phase, which gave rise to the adult level of TTX-S NaChs, was dependent upon the presence of innervation. This phase would correspond to changes seen after two weeks of regeneration after notexin poisoning.

The cellular signal(s) that allows innervation to control expression of the two NaCh isoforms has not yet been identified. Experiments on cultures of adult mouse diaphragm and embryonic mouse spinal cord led Ziskind and Harris (1979) to conclude that this signal was independent of muscle electrical activity. The nerve could regulate Schwann cell metabolic activity or the stability of basal lamina molecules. Alternatively, the nerve could be a source of diffusible factors. Recently, a number of diffusible factors affecting the expression and synthesis of AChRs and NaChs have been identified, including calcitonin gene-regulated peptide (CGRP; New and Mudge, 1986; Laufer and Changeux, 1987) and acetylcholine receptor–inducing activity (Usdin and Fischbach, 1986; Falls et al., 1990). CGRP increases the level of intracellular cAMP (Falls et al., 1990), which has been shown to increase NaCh expression in muscle cells (Catterall, 1992), but the effect of CGRP on NaChs has not yet been directly studied. Acetylcholine receptor–inducing activity has been shown to increase functional NaChs 3–4-fold on cultured muscle cells, although it has no effect on intracellular cAMP levels (Falls et al., 1990; Corfas and Fischbach, 1993). Either of these molecules could be the trophic factor released by nerve terminals that regulates expression of the two NaCh isoforms.

In conclusion, these experiments indicate that the presence of innervation is not required for NaCh aggregation to occur at regenerating neuromuscular synapses. This leaves only the Schwann cells or basal lamina molecules as possible sources for the signal inducing synaptic aggregation of this protein. One remaining question is how this relates to the increase in synaptic NaCh density that normally occurs during postnatal development. Is this a process that occurs independently of AChR clustering, for example, or must it be viewed as one aspect of a program of synaptic development initiated by agrin, with each subsequent step dependent on previous ones for the program to cascade forward to full synaptic maturation? The answer to this question will provide a better understanding as to how synapses are constructed and how they function.

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